CHARACTERISATION AND BIOLOGICAL ACTIVITIES OF *TINOSPORA CRISPA* (MENISPERMACEAE) EXTRACT WITH EMPHASIS ON ALKALOIDS

HAZRULRIZAWATI BT ABD HAMID

Thesis submitted in fulfillment of the requirements
for the award of the degree of
Doctor of Philosophy of Science in Industrial Chemistry

Faculty of Industrial Sciences and Technology
UNIVERSITI MALAYSIA PAHANG

JULY 2013

ABSTRACT

A study was carried out on the characterisation and biological activities of *Tinospora crispa* extract with emphasis on alkaloids. Methanolic extraction, crude alkaloid fractionation and separation using different chromatographic techniques (open columns and preparative TLC) were used for isolation of pure compounds. The structure of the isolated compounds were elucidated by spectroscopic methods such as UV, IR, 1D (^{1}H, ^{13}C, DEPTQ), 2D (COSY, HMQC, HMBC) NMR, MS and also by comparison with the literature. Twelve compounds were isolated from these plants comprising eight alkaloids and four nonalkaloid compounds. Six of the alkaloid, viz., *N*-formylannonaine, *N*-formylnornuciferine, lysicamine, magnoflorine, columbamine and dihydrodiscretamine previously isolated from *T.crispa* and other species the genus *Tinospora*. In addition, one known alkaloid, liriodenine was found for the first time in this study. A novel alkaloid, 4,13-dihydroxy-2,8,9-trimethoxydibenzo[a,g]quinolizinium was isolated and its structure was established by modern spectroscopic technique. A biosynthethic route to this alkaloid is proposed. Another new compound isolated was aliphatic amine, *N,N*-dimetylhexadecan-1-amine. Three types of bioactivity studies, viz., acetylcholinesterase inhibitory, radical scavenging and antimicrobial studies were carried out on isolated compounds containing nitrogen. The quaternary protoberberine alkaloids; columbamine and dihydrodiscretamine were found to be potent acetylcholinesterase inhibitors. Most of the isolated quaternary alkaloids showed moderately (IC_{50} > 500-800 μg/mL) radical scavenging activity. Oxaporphine alkaloids, lysicamine, liriodenine and aliphatic amine inhibited the growth of the *Gram*-positive bacteria, *Staphylococcus aureus* (+) and *Enterococcus faecalis* (+). Aliphatic amine also exhibited inhibitory activity against *Gram*-negative bacteria, *Proteus vulgaris* (-) and *Psuedomonas aeurogenosa* (-). Results of the present biological activity investigation further points to the potential of this plant species as a good source of new AChE inhibitors. The antioxidant and antimicrobial properties of different compounds support documented traditional use of *T. crispa* in wound healing and treatment of rheumatic, diarrhoea, ulcers, itches and wounds.

ABSTRAK

Satu kajian telah dijalankan terhadap pencirian dan aktiviti biologi ekstrak pokok *Tinospora crispa* dengan tumpuan kepada alkaloid. Ekstrak methanol, pemisahan campuran alkaloid dan kaedah-kaedah kromatografi (kromatografi kolum gravity dan kromatografi lapisan nipis persediaan) telah digunakan dalam proses pengasingan alkaloid. Struktur-struktur alkaloid tersebut dikenalpasti menggunakan kaedah spektroskopi seperti spekroskopi ultra lembayung, spektroskopi inframerah, spekroskopi 1D RMN, spekroskopi korelasi RMN, spektroskopi jisim serta perbandingan data dengan tinjauan kajian. Dua belas sebatian telah diasingkan yang terdiri daripada lapan sebatian alkaloid dan empat sebatian bukan alkaloid. Enam daripada sebatian alkaloid, *N*-formylannonaine, *N*-formylnornuciferine, lysicamine, magnoflorine, columbamine dan dihydrodiscretamine telah dilaporkan pengasingannya daripada *T.crispa* dan spesis lain daripada genus *Tinospora*. Tambahan satu sebatian alkaloid yang telah dikenalpasti iaitu liriodenine telah diasingkan buat pertama kali dalam pokok ini. Satu sebatian alkaloid baru, 4,13-dihydroxy-2,8,9-trimethoxydibenzo[a,g]quinolizinium telah diasingkan dan dibuktikan dengan kaedah spektroskopi moden. Penghasilan alkaloid ini secara biosynthesis turut dicadangkan. Satu lagi sebatian baru adalah sebatian amina rantai lurus, *N*,*N*-dimetylhexadecan-1-amine. Tiga jenis aktiviti biologi telah dijalankan ke atas sebatian yang mengandungi elemen nitrogen iaitu, perencatan enzim acetylcholinesterase, aktiviti perangkap radikal bebas serta aktiviti antimikrobial. Alkaloid kuarterner protoberberine; columbamine and dihidrodiscretamine didapati berpotensi sebagai salah satu perencat enzim acetylcholinesterase. Hampir semua sebatian kuaternar alkaloid menunjukkan aktiviti sederhana (IC50 > 500-800 μg/mL) sebagai perangkap radikal bebas. Alkaloid daripada kumpulan oxoaporphine, lysicamine, liriodenine dan sebatian amina rantai lurus didapati merencatkan pertumbuhan bakteria *Gram*-positif; *Staphylococcus aureus* (+) and *Enterococcus faecalis* (+). Sebatian amina rantai lurus juga menujukkan perencatan aktiviti bagi *Gram*-negatif bakteria; *Proteus vulgaris* (-) dan *Psuedomonas aeurogenosa* (-). Hasil kajian aktiviti biologi memberi petunjuk akan potensi pokok ini sebagai satu sumber baru sebagai perencat enzim acetylcholinesterase. Aktiviti antioksida dan antibakteria yang ditunjukkan oleh sebatian yang berbeza adalah sebagai sokongan kepada penggunaan pokok *T.crispa* secara tradisional dalam merawat sakit sendi tulang, cirit birit, ulser, kegatalan dan luka.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

β	beta
δ	chemical shift
°C	degree Celsius
cm^{-1}	per centimeter
υ	frequency of the wave
γ	gamma
g	gram
Hz	hertz
k	kilo
L	liter
λ	wavelength
λ_{max}	maximum wavelength
mg	milligram
mL	milliliter
mM	millimolar
M	molar
µ	micro
µL	microliter
%	percentage
ppm	part per million
U	unit
UmL^{-1}	unit per milliliter

LIST OF ABBREVIATIONS

AChE	acetylcholinesterase enzyme
ATCI	acetylthiocholine iodide
^{13} C NMR	carbon nuclear magnetic resonance
CC	column chromatography
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
$CHCl_3$	chloroform
COSY	correlation spectroscopy
d	doublet
dd	doublet of doublet
ddd	doublet of doublet of doublet
DEPTQ	distortionless enhancement by polarization transfer with retention of quaternaries
DPPH	2,2,-diphenyl-1-picrylhydrazyl
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
EIMS	electron impact mass spectrometry
EtOAc	ethyl acetate
EtOH	ethanol
GCMS	Gas Gromathoraphy-Mass Spectroscopy
HCl	hydrochloric acid
^{1}H NMR	proton nuclear magnetic resonance
HMQC	heteronuclear multiple quantum coherence
HSQC	heteronuclear single quantum coherence
IC_{50}	concentration providing 50% inhibition
IR	infrared
J	coupling constant
KBr	potassium bromide
m	multiplet
M^+	molecular ion
MeOH	methanol
$MgCl_2$	magnesium chloride

MHz	megahertz
MIC	minimum inhibitory inhabitation
MS	mass spectrometry
m/z	mass to charge ratio
NaCl	sodium chloride
NH_4OH	ammonium hydroxide
NIST	National Institute of Standards and Technology
NMR	nuclear magnetic resonance
pH	power of hydrogen
PTLC	preparative thin layer chromatography
R_f	retention factor
RP-18	reverse phase silica gel
s	singlet
SiO_2	silica gel
t_R	retention time
t	triplet
TIC	total ion current chromatogram
TLC	thin layer chromatography
UV	ultraviolet

CHAPTER 1

1.1 BACKGROUND

The importance of natural products, particularly those derived from plants as a source of molecular diversity for drug discovery research and development may seem obvious. Historically, a number of recent reports have provided information about the importance of natural products as a source of bioactive compounds. Plants have good reason to produce bioactive substances. This is probably related in large part to the fact that they do not move, and therefore defend themselves by repelling or killing predators, that comprise insects, microorganisms, animals, or even other plants. Plants have evolved a complex chemical defense production system, and this may involve a large number of different chemical compounds. A great advantage of the natural products drug discovery approach is that it is capable of delivering complex molecules that are not accessible by other routes. In addition, it can provide templates leads for future drug design (Ibrahim, 2007).

Traditional plant-based medicines historically used in different parts of the world or different cultural systems are considered as “alternative medicine”. Since these plants, used singly and/or in combination with other botanicals and ingredients, have been a part of which cultural pharmacopeia and primary health care, they may provide new leads for modern medicine and new chemical entities. In many parts of the world such as Africa and Asia, the plants traditionally used for health care and medicine are still as important today as they have been previously as the only health care options affordable (Welch, 2010).

A number of traditional medicines have been scientifically proven efficacious and several have led to new mechanisms of therapeutic action against cancer,

inflammation, autoimmune diseases, Alzheimer's disease, Parkinson's disease, malaria, and cardiovascular disease (Kong et al., 2003). Certain areas of drug therapy depend largely on bioactive compounds derived from natural product and natural product mimics, in particular, antibacterial, anti-infectives and antihypertensive drugs (Cos et al., 2003). Natural product chemistry is an area of chemistry that has successfully delivered a large number of antimicrobial agents, anticancer and antiviral drugs that have been promoted to the commonly prescribed drugs (Kong et al., 2003).

Today, natural product chemists are involved in performing phytochemical studies on plants having biomedical importance in folk medicine history. These studies have yielded several biomedical agents including paclitaxel and emetin. Paclitaxel (Taxol®), which was isolated from bark extract of the Pacific yew tree, (*Taxus brevifolia*) , is an important drug used in the treatment of cancer (Burstein et al., 1992). It is an effective drug of choice for the treatment of lung, ovarian and breast cancer. Similarly, the isoquinoline alkaloid, emetine isolated from *Cephaelis ipecacuanha,* has been used for many years for the treatment of abscesses caused by the spread *of Escherichia histolytica* infections (Chang and But, 1986).

Drug discovery from plants has evolved to include numerous fields of inquiry and various methods of analysis. The process typically begins with a botanist, ethnobotanist, ethnopharmacologist or plant ecologist collecting and identifiying plants of interest. Phytochemists then prepare extracts from the plant material, subject these extracts to biological screening in pharmacologically relevant assays and commence the process of isolation and characterization of the active compounds through bioassay-guide fractionation (Balunas and Kinghorn, 2005).

The investigation of bioactive natural product was mainly concerned with the study of discovering bioactive constituents from plant and living organisms. During the early stages of natural product research, detection, isolation and structural elucidation of natural product were the main focus of researchers. Nowadays, the advent of more efficient methods of isolation, separation, purification and the availability of new bioassay techniques have greatly stimulated the development of research in this field (Vlietinck, 1998).

Tinospora crispa (family: Menispermaceae; Malay: akar seruntun, patawali) is widely used in Malay traditional medicine as well as other indigenous peoples in Malaysia as an ethno-remedy for the treatment of hypertension and diabetes (Dweck and Cavin, 2006). Besides that, *T. crispa* is also used to treat tooth and stomach aches, coughs, asthma and pleurisy (Rahman et al., 1999). Scientifically, *T. crispa* has been demonstrated to possess antibacterial (Zakaria and Matjais, 2006), antiflarial, antimalarial, antipyretic and antihyperglycaemic activities (Kongkathip et al., 2002). Based on this ethanomedical importance of plants, the present study is designed to explore the active phytochemicals from the plants.

The plant family Menispermaceae has long been a rich source of alkaloids, terpenoids and glycosides. *Tinospora* is a genus within Menispermaceae reputed for it medicinal properties (Pathak et al., 1995). The great majority of compounds isolated from *Tinospora* species have been furanoid diterpenes of the clerodane type, and their glycoside derivatives (Hungerford et al., 1998). More than 50 clerodane-based compounds have been isolated from various *Tinospora* species. Quaternary alkaloids are the major alkaloid type isolated from *Tinospora* species, and these are mostly of the protoberberine. Some non-quaternary alkaloids have also been isolated from certain *Tinospora* species and most recently the *N*-acyl aporphine alkaloids (Hungerford et al., 1998). The medicinal value of *Tinospora* species can most probably be attributed to the wide variety and high concentrations of alkaloids.

Alkaloids constitute one of the most important natural products produced in the plant kingdom. Alkaloids are organic nitrogenous bases found mainly in plants, but also to a lesser extent in microorganisms and animals. Several alkaloids in popular use include caffeine; a psychostimulant is largely obtained from the decaffeination of *Coffea* species and codeine as an antitussive. Cocaine is used as a local anesthetic (Roberts and Wink, 1998). Morphine is an indispensable analgesic used for treatment of severe pain. Quinine is noted for its antimalarial activity and remains on the market as an antipyretic (fever suppressant), although its earlier dominance by synthetic drugs such as quinoline derivatives include chloroquine, amodiaquine, quinine, quinidine, mefloquine, primaquine, lumefantrine and halofantrine (Travassos and Laufer, 2009).

1.2 PROBLEM STATEMENT

The chemical constituents of *T. crispa* extracts have been extensively studied since the 1980s. The major active ingredients of *T. crispa* are identified as terpenoids and terpenoid glycosides. The terpenoid glycosides are mainly composed of borapetosides A, B, C, D, E and F (Cavin et al., 1998; Choudhary et al., 2010; Kongkathip et al., 2002; Martin et al., 1996; Pachaly and Schneider. 1992 and Pathak et al., 1995). Recently, Choudhary et al. (2010) reported the isolation of a new aporphine alkaloid, *N*-formylasimilobine 2-O-β D-glucopyranoside, along with nine known alkaloids from stems of *T. crispa*. Although the same species had been studied by researchers from Malaysia, China, Pakistan, India and Thailand, different localities or environment probably give variations of constituents because of geographic distribution, climate, different plant parts and morphology as well as ecological conditions which influence the biosynthesis of secondary metabolites of the plants. There are many more bioactive compounds waiting to be isolated. *T. crispa* is widely used in traditional medicine; however there are only a few reports that indicate which chemical compounds contribute to the medicinal properties of the plant (Ruan et al., 2012). *T. crispa* is often used as tonic plants due to its bitter taste. This can be attributed to the high concentration of alkaloids in these plants. Many alkaloids are pharmacologically active substance (Robert and Wink, 1998).

1.3 RESEARCH OBJECTIVES

This research is directed towards the study of *T. crispa* extract including the isolation, identification and characterisation of the compounds with emphasis on alkaloids as well as the biological activities. The specific objectives of this research are to:

1. To isolate compounds with emphasis on alkaloids in *T. crispa*.
2. To elucidate the structure of compounds by spectroscopic techniques.
3. To evaluate for radical scavenging activity, acetylcholinesterase inhibitory activity and antimicrobial activity.

1.4 SCOPE OF THE STUDY

There are two main approaches in natural product researches including chemical investigations and the bioactivity studies. Specifically, the chemical compounds of the alkaloid extracts were purified by chromatographic techniques and followed by recrystallization. Characterisation of isolated compounds was carried out by spectroscopic methods such as IR, NMR (^{1}H, ^{13}C, DEPTQ, COSY, HMQC, HMBC) and MS.

Evaluation of the biologically activities of the isolated compounds were carried out using more than one technique to cover the bioactivity of interest. The isolated compounds were screened for radical scavenging activity, acetylcholinesterase inhibitory activity and antimicrobial activities.

CHAPTER 2

LITERATURE REVIEW

2.1 BOTANICAL OVERVIEW

Tinospora crispa is classified under family Menispermaceae of the order Ranunculales (Figure 2.1).

Kingdom	: Plantae
Division	: Angiospermae
Class	: Magnoliidae
Order	: Ranunculales
Family	: Menispermaceae
Genus	: Tinospora
Species	: *Tinospora crispa*

Figure 2.1: Specific classification of species *Tinospora crispa*

Source: Wang et al. (2007)

2.1.1 Menispermaceae

The Menispermaceae is composed of 71 genera and about 520 species (Jacques and Zhou, 2010). The family Menispermaceae is highly specialized in that its extraordinarily rich diversification of bisbenzylisoquinoline and aporphine derivatives.

Up to the end of 1996, 1858 alkaloids have been described from 244 species of the family (Barbosa-Fillo et al., 2000). These alkaloids include many important discoveries in the field of medicine and pharmaceutically active compounds (Dewick, 2002). A literature survey revealed that twenty-one genera are used for medicinal purposes in the world. Nine genera in use are *Cissampelo*s, *Cocculus*, *Dioscoreophyllum, Jateorhiza*, *Sphenocentrum*, *Stephania*, *Tiliacora*, *Tinospora* and *Triclisia* (De Wet, 2006).

Menispermaceae are twinning or rarely erect shrubs or lianas; rarely herbs or trees. Wood in cross-section showing broad, medullary rays. The leaves are alternate, spiral, petiolate. Flowers of Menispermaceae are dioecious. (De Wet, 2006). Seeds often curved and horseshoe-shaped, with uniform or ruminate endosperm or without endosperm. Embryo straight or curved; cotyledons flat or more or less terete, foliaceous or fleshy, divaricate or appressed (Kessler, 1993).

2.1.2 Genus *Tinospora*

Genus *Tinospora* consists of approximately 35 species where 23 are found in Asia, Australia and the Pacific, two in Madagascar, seven in tropical Africa and three in southern Africa (De Wet, 2006). Species of the *Tinospora* genus have prominent roles in the traditional medicinal practices of Australia, Africa and Asia (Hungerford et al., 1998).

Tinospora smilacina Benth. (Menispermaceae), commonly known as `snakevine', is a semideciduous woody climber which inhabits open forests and low, open woodlands across northern Australia and the east coast to northern New South Wales. The stems, leaves and roots of this species have been used in traditional aboriginal medicine for treatment of a remarkably wide range of disorders. In particular, the roots were used to counter animal stings and used in Western Australia spesifically as a snakebite remedy (Hungerford et al., 1998).

Tinospora cordifolia is an official herb in the Indian Pharmacopoeia and also in the Ayurvedic Pharmacopoeia. The stems of *T. cordifolia* (TC) known as Guduchi

vernacular name, is widely used in Ayurveda as Rasayana to enhance general body resistance, promote longevity and as antistress and adaptogen (Patil et al., 2009).

Tinospora rumphii Boerl. (synonyms *T. tuberculata* Buemee and *T. crispa* Miers) is a climbing vine with stems rich in warts. It is widely distributed in the Philippines and in some Southeast Asian countries. This bitter tasting plant, known in the Philippines as Makabuhai, is used for the treatment of stomach troubles, ulcers and fevers, as a tonic and a febrifuge for malaria and smallpox, as a vulnerary for itches and wounds, and many other purposes (Martin et al., 1995).

The Chinese Materia Medica recorded 4 major *Tinospora* species (*Tinospora sagittata*, *Tinospora sinensis*, *Tinospora crispa* and *Tinospora capillipes*). Traditional Chinese Medicines (TCM) products like the famous Fufang Danshen tablets is prepared from the roots of *Tinospora sagittata* (Oliv.) Gagnep and *Tinospora capillipes* Gagnep. (Shi et al., 2007).

Overall the genus *Tinospora* has been cited four times as analgesic, fourteen times for anti-malarial property and sixteen times for diabetes treatment (De Wet, 2006). *Tinospora* is mostly used in the rest of the world as an anthelmintic, treatment of arthritis and rheumatism, diabetes, fever, malaria, wounds, ulcers and as tonic (De Wet, 2006). Their potential in ethnopharmacology and drug discovery should not be underestimated.

2.1.3 *Tinospora crispa*

Tinospora crispa (L.) Miers ex Hook. f. &Thoms, known by different botanical synonyms such as *Tinospora rumphii* or *Tinospora tuberculata*, belongs to the family Menispermaceae (Martin et al, 1995) *T. crispa* is also known as “patawali” or “akar seruntum” in Malay, “boraphet” in Thai, “makabuhay” in Philipines, “day coc” in Vietnamese and Bo Ye Qing Niu Dan in Chinese (Fukuda et al., 1983). It is found in primary rainforests or mixed deciduous forests throughout a large part of Asia and Africa (Pathak et al., 1995).

T. crispa is a climbing, dioeciously vine reaching a height of 4-10 meters. The stem is about 1 centimeter thick, somewhat fleshy, with scattered protuberances. The leaves are thin, ovate, 6-13 centimeters long and 6-13 centimeters wide. The petiole is 5-15 centimeters long. Inflorescences racemose, unbranched or occasionally shortly branched, appearing before leaves, flowers 2- or 3-fascicled. Male inflorescences very slender, 5-10 cm or longer. Male flowers: sepals 6 in 2 whorls, green, glabrous, petals 3-6, yellow, obovate-spatulate. Female inflorescences 2-6 cm, flowers mostly 1 per node. Female flowers: sepals and petals as in male; stigma lobes very short.Fruiting peduncle 15-20 mm; carpophores 2-3 mm (Hooker and Thomson, 1855).The botanical features of *T. crispa* is depicted in Figure 2.2**.**

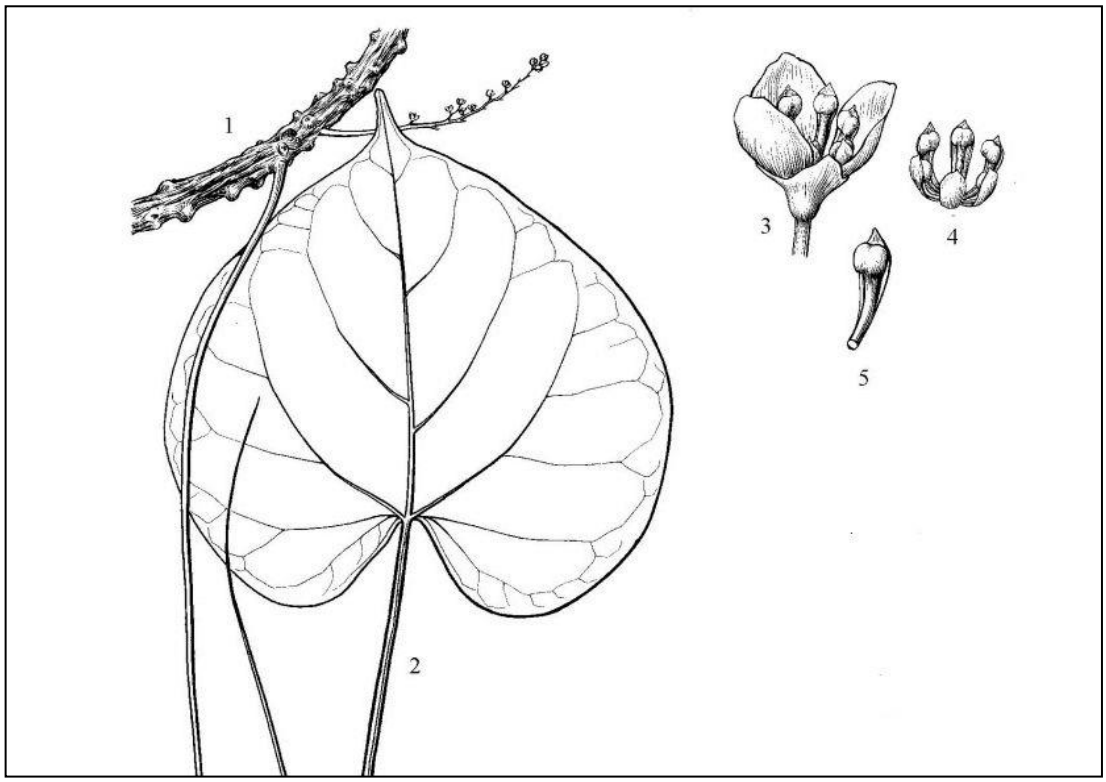

Figure 2.2: *Tinospora crispa*, 1.Flowering branch with aerial root, 2.Leaf, 3.Male flower, 4.Male flower with sepal remove, 5.Stamen

Source: Hooker and Thomson (1855)

2.2 ETHNIC MEDICAL USAGE

In Malaysia, an aqueous extract of *T. crispa* stems is taken orally to treat diabetes mellitus (Noor and Ashcroft, 1989). The young stem of the plant is chewed to relieve hypertension, toothache and abdominal pains (Zaridah et al., 2001). Extracts of *T. crispa* roots pounded with garlic and a pinch of salt is drunk once per day for 3 days for filarial eradication. Poultice made by pounding *T. crispa* root with rice wash and charred coconut husks was used to cure rheumatism (Ahmad and Raji, 1993).

In Thailand, the stem of *T. crispa* is one of the most popular traditional appetizer and for febrifuge for malaria and smallpox. According to the Thai, it makes the blood "bitter and cool" (Fukuda et al., 1983).

T. crispa is known to the Filipinos as "makabuhay" meaning 'that which bring back life' due to their believed that it can cure malarial fever (Salazar et al., 1987). In the Philippines it is also used for the treatment of stomach troubles, ulcers and as a vulnerary for itches and wounds (Martin et al., 1995).

2.3 PHARMACOLOGICAL IMPORTANCE

Various studies have been conducted on the pharmalogical effectiveness of *T. crispa* extracts to support ethnopharmacological claims.

The antihyperglycaemic and insulinotropic effect of *T. crispa* observed in *in vivo* and *in vitro* experimental models supports the anecdotal claims for its antidiabetic activity (Noor and Ashcroft, 1989). This could lead to the formulation of a novel drug for the treatment of noninsulin-dependent diabetes mellitus. However, vigorous characterisation of the extract with respect to its mechanism of action is necessary before a pharmacological role can be assigned. The results clearly showed that the antihyperglycaemic effect is not due to interference with intestinal glucose uptake or uptake of the sugar into the peripheral cells. Rather, the antihyperglycaemic effect of *T. crispa* is probably due to stimulation of insulin release via modulation of intracellular Ca^{2+} concentration in pancreatic β-cells (Noor and Ashcroft, 1998). That the